



## A general method for two-dimensional protein electrophoresis of fruit samples

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### Abstract

During experiments characterizing and identifying proteins from controlled atmosphere-stored apple and peach fruit, we optimized methods for the extraction and two-dimensional electrophoresis (2-DE) of fruit proteins, using commercially available immobilized pH gradient strips for the first dimension. The method is relatively rapid with minimal handling of small amounts of sample, and has been reproduced successfully for 2-DE of a variety of fruit and plant tissues in our labs. Critical factors for fruit tissues include using acetone precipitation following incubation in a lysis buffer, and a long iso-electric focussing time. We have observed no interference to focussing from such troublesome fruit components as soluble pectins, polyphenolics, or high-acidity fruit, using this protocol. In addition we have used the method with no modification, for a range of fruit tissues including low protein sources (apple and peach flesh), high lipid material (avocado fruit flesh) and high acidity lemon tissue. As the method in our hands is straightforward and robust, we recommend the method for routine 2-DE separations of fruit samples. Published by Elsevier B.V.

**Keywords:** 2-DE; Varied plant tissues; Rapid extraction; Mini-prep

### 1. Introduction

Horticulture researchers have long been interested in biochemical characterisation of fruit before, during

and after storage (e.g. Lay-Yee et al., 1990; Watkins et al., 1997; Abdi et al., 2002; Obenland et al., 2003). In order to accomplish a more comprehensive understanding of the postharvest physiology of fruit,

**Abbreviations:** 2-DE, two-dimensional electrophoresis; BSA, bovine serum albumin; CHAPS, 3(3-[3-chloramidopropyl]dimethylammonio)-1 propanesulfonate; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis(beta-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]; IEF, isoelectric focussing; IPG, immobilised pH gradient; PAGE, polyacrylamide gel electrophoresis; PDA, piperazine diacrylamide; PEG, polyethylene glycol; PVDF, polyvinylidene fluoride; PVPP, polyvinyl polypyrrolidone; SDS, sodium dodecyl sulphate; TEMED, *N,N,N,N'*-tetramethyl ethylenediamine; TCA, trichloroacetic acid

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we have developed methods to characterize fruit protein expression differences using two-dimensional electrophoresis (2-DE).

Two-dimensional gel electrophoresis has proven itself as a powerful technique to separate proteins based on charge and molecular weight. The technique is widely used in studies of animals and microorganisms, and has been used successfully in separating plant proteins (Granier, 1988; Dominguez-Puigjaner et al., 1992; Chang et al., 2000). However, we found fruit tissues difficult to process through 2-DE using these established methods, presumably because fruit have many compounds that interfere with the extraction and 2-DE of proteins. These include phenolics, pectins, highly acid components, and proteinases. In addition, the presence of cell walls makes plant tissues more difficult to finely grind to maximize protein extraction than animal tissues. Consequently, extraction of plant tissue often requires special techniques and modifications over that used for animal tissues and microorganisms to avoid these problems, including extraction with phenol (Peck et al., 2001; Abdi et al., 2002) and SDS (Santoni et al., 1998), and concentrating the extracts with trichloroacetic acid (Damerval et al., 1986). Consequently, we developed modifications of these methods for fruit which we have found work well with a wide variety of fruit tissues.

The simple method reported in this paper, has given reproducible 2-DE results for a variety of fruit tissues, including cortex from apple, avocado, and peach as well as lemon flavedo and avocado skin. In addition, we have found it successful in other plant tissues, including *Arabidopsis* leaves, apple cells growing in culture, kiwifruit meristems and *Pinus radiata* xylem and needle tissue. There are no phenol or TCA requirements, no SDS in the first dimension, and most steps are performed at room temperature.

## 2. Materials and methods

In brief, the sample is ground in liquid nitrogen and extracted at room temperature with a buffer containing multiple chaotropes (denaturing salts which bind strongly to proteins). The protein is then precipitated by acetone incubation, resolubilized and isoelectri-

Table 1  
Recipes for selected steps in 2D electrophoresis

Component		Source or comment
Chemical	Concentration	
Rehydration solution		
Urea	7 M	Bio-Rad
Thiourea	2 M	BDH AnalaR
DTT	65 mM	Sigma
CHAPS	4% (v/v),	Bio-Rad
IPG buffer	2% (v/v)	Amersham (Table 2)
Bromophenol blue	a few grains	Bio-Rad
Lysis buffer		
Urea	7 M	
Thiourea	2 M	
Tris	40 mM	Gibco
DTT	75 mM	
CHAPS	4% v/v	
Equilibration solution		
Urea	6 M	
Tris	50 mM	
Glycerol	30% (v/v)	BDH
SDS	2% w/v,	Applichem
Bromophenol blue	trace	
Adjust to pH 8.8		
PAGE gel		
Acrylamide solution	13.9% (v/v)	Bio-Rad
PDA	1.24% (w/v)	Bio-Rad
Tris	0.52 M	
Sucrose	0.8 M	BDH AnalaR
Sodium thiosulphate	0.007 M	BDH AnalaR
Ammonium persulphate	0.07% (w/v)	Bio-Rad
TEMED	0.07% (w/v)	Bio-Rad

cally focussed on an IPG strip followed by SDS-PAGE electrophoresis.

The first dimension is run on commercial Amersham Pharmacia IPG dry strips and the second dimension on laboratory-prepared vertical SDS-PAGE gels. The method below is given in order of procedure.

### 2.1. Strip rehydration

Amersham Pharmacia Biotech IPG strips were rehydrated overnight, at room temperature, under paraffin oil in volumes according to the manufacturers instructions, and in the Amersham Pharmacia Biotech drystrip reswelling tray, with the rehydration solution (Table 1). Strips were always allowed to rehydrate overnight, under paraffin oil, before use.

## 2.2. Tissue preparation and protein extraction

Fresh or frozen fruit tissue was ground in a small mortar and pestle in liquid nitrogen. The powdered sample (0.2 g) was immediately transferred to another mortar containing 1.0 ml lysis buffer (Table 1). The sample and buffer were thoroughly mixed and transferred to a 1.5 ml Eppendorf tube at room temperature for at least 1 h before further processing. For samples containing phenols, such as leaf or fruit material, 0.1 g insoluble PVPP (Sigma) was added at the time of extraction. The lysed extract was centrifuged at  $10,000 \times g$  for 15 min at room temperature, and the supernatant added to three volume of ice cold 100% acetone. The solution was allowed to precipitate at  $-20^\circ\text{C}$  for 2 h. The acetone precipitate was then centrifuged for 15 min at  $10,000 \times g$  and the supernatant discarded. The pellet was air-dried and then resolubilised in 200  $\mu\text{l}$  of the rehydration buffer used to re-swell the IPG strips. If the sample was still viscous at this point, a further 200  $\mu\text{l}$  rehydration buffer was added. A protein assay (Bradford, 1976), was used to standardise protein loadings for the first dimension. In order to avoid detergent interference in the assay, the sample was highly diluted (10  $\mu\text{l}$  resolubilized sample, 790  $\mu\text{l}$  dH<sub>2</sub>O, 200  $\mu\text{l}$  Bio-Rad protein reagent concentrate) and BSA standards were dissolved in the same rehydration buffer.

## 2.3. PAGE

The first dimension was run essentially as described in the Amersham Biosciences manual for 2D methods (Berlkeman and Stenstedt, 1998) with important modifications. A 50  $\mu\text{l}$  aliquot of suitably diluted protein (Table 2) was applied through sample-cup loading at the cathodic end of the strip,

and the pre-programmed power supply (EPS 3500XI, Amersham Pharmacia Biotech) started immediately. The voltage was changed over a linear gradient from 0 to 200 V over 0.01 h followed by 200 V for 1 h, then the voltage was increased linearly to 3500 V over 1 h and then run at 3500 V (Table 2) at  $20^\circ\text{C}$ .

After completion of the isoelectric focussing, the strips were equilibrated for 12 min in 10 ml of equilibration solution (Table 1) to which 100 mg of DTT had been added. Strips were then equilibrated for 6 min in 10 ml equilibration solution (Table 1) containing 400 mg iodoacetamide (Sigma). Alternatively, focussed strips could be stored at  $-80^\circ\text{C}$  for up to 2 months.

Following equilibration, strips were run on a SDS-PAGE Protean II unit (Bio-Rad) using laboratory-made gels (Table 1). No degassing was required. Gels were always poured the night before use and left to polymerise at room temperature, under a 50% isopropyl alcohol overlay. Distilled water replaced the alcohol once the gel surface had set after approximately 1 h. Broad-range prestained molecular weight standards from Bio-Rad (4  $\mu\text{l}$ ) were applied to one small (1  $\times$  2 mm) ‘application piece’ of filter paper for each gel. A drop of boiling-hot agarose (0.05% agarose in tank buffer) was added to each stained piece to seal the markers into the paper. Once set, the application piece was loaded into place, beside the strip at the cathodic end. Hot agarose was pipetted onto the surface of the second dimension gel, and the focussed IPG strip was loaded through the liquid and gently pressed onto the surface of the second dimension gel to ensure good contact. A tank buffer of 25 mM Tris, 192 mM glycine (Bio-Rad), 0.1% SDS was used for the second dimension. Gels were run at 24 mA/gel for 4.5 h, with cooling from continuous running of tap water through the Protean

Table 2  
Conditions used during electrofocussing

IPG strip type Amersham Pharmacia Biotech	IPG buffer Amersham Pharmacia Biotech	Optimal protein loading ( $\mu\text{g}$ protein/strip)	Focussing duration (V h)
pH 3–10 NL 7 cm (catalogue number 17-6001-12)	PH 3–10 NL (catalogue number 17-6000-88)	2–6	98000
pH 3–10 NL 18 cm (catalogue number 17-1235-01)	PH 3–10 NL	15–30	140000
pH 4–7 18 cm (catalogue number 17-1233-01)	PH 4–7 (catalogue number 17-6000-86)	30–60	175000
pH 6–9 18 cm (catalogue number 17-6001-88)	PH 6–11 (catalogue number 17-6001-78)	60–120	175000

Note that the focussing duration is calculated during the constant 3500 V stage.

II cooling core. Staining was performed by placing gels into a fixative solution (10% methanol, 7% acetic acid) for 30 min and then to Sypro Ruby (Molecular Probes) for 3 h or overnight. The Sypro stained gels were digitally imaged (GelDoc, BioRad) under UV excitation, and the image analysed on ImageMaster 2D Elite software (Amersham Pharmacia Biotech). After Sypro staining, samples were stained with a modified colloidal coomassie stain (Neuhoff et al., 1988) (17% ammonium sulphate, 3% phosphoric acid, 34% methanol, 0.1% Coomassie G-250), and dried on glass plates at room temperature under cellophane, and stored. Alternatively for Western analysis, gels were prepared for transfer to PVDF membrane (Towbin et al., 1979), with a modification, using a buffer of 0.192 M glycine, 0.025 M Tris, pH 8.3, 0.1% SDS and 20% methanol. A Bio-Rad semi-dry transfer apparatus was used for transfer at 5 V for 4.5 h.

### 3. Results and discussion

The method described here is robust, simple, straightforward and reproducible for a wide range of fruit tissues from postharvest storage experiments. We show examples of routine 2-DE gels of separated fruit proteins from several sources (Fig. 1) that illustrate the versatility and resolution of the method which works well on both broad range (pH 3–10) and narrow range strips (e.g. pH 4–7). We developed this modified method of 2-DE because we found previously published methods gave unsatisfactory results, especially on commercial IPG strips.

To develop the method, we compared a range of sample extraction protocols. These included methods described by Ryan et al., (1988) (aqueous sample extraction resulting in smeared results for 2-DE), Pridmore et al., (1999) (TCA and a

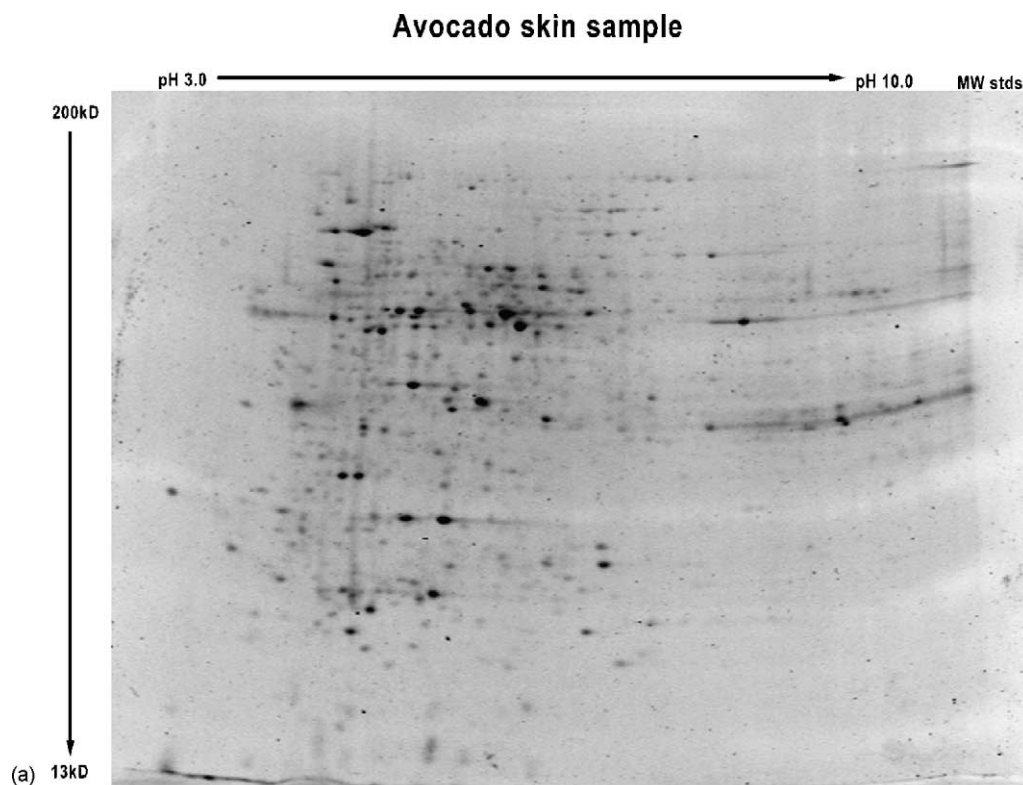


Fig. 1. Typical examples of 2-DE of different extracts of plant samples run under the methods described in this paper. (A) Avocado skin extract (Sypro Ruby stain) pH 3–10 NL 18 cm strip. (B) Royal Gala apple fruit flesh cell culture extract (colloidal coomassie stain) pH 3–10 NL 18 cm strip. (C) Braeburn apple fruit flesh extract (Sypro Ruby stain) pH 3–10 NL 18 cm strip.

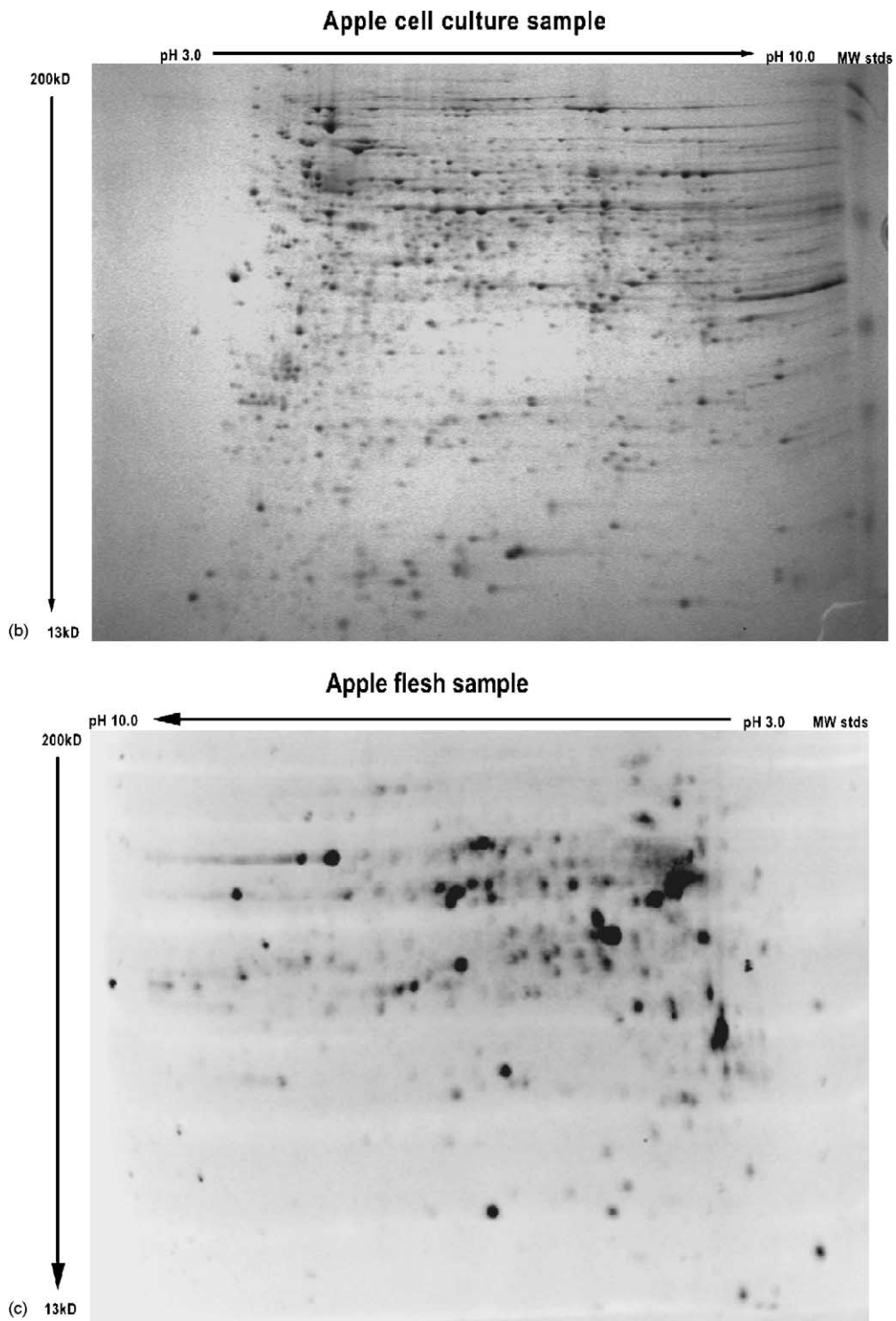


Fig. 1. (Continued).

protease inhibitor; very little focussing), Horst et al., (1980) (potassium carbonate buffer; streaky gels) and Dominguez-Puigjaner et al., (1992) and Abdi et al., (2002), (phenol extraction, which we prefer to avoid due to the toxic nature of the chemical, and which gave a blurred background on sypro staining). Results from our modified protocol gave abundant, well-focussed, well-separated spots, with no smearing or streaking of spots (Fig. 1).

Most of these methods utilized a precipitation step to concentrate the proteins and to clean up the sample from salts, lipids and sugars. This appeared to be especially important when dealing with dilute fruit proteins. For example, a published standard 2-DE protocol (Damerval et al., 1986) used TCA as the protein precipitant for fruit tissue samples, but we found dilute fruit proteins did not resolubilize after TCA precipitation. Acetone precipitation worked well, although resolubilization of proteins is more difficult if the acetone is warmer than ice-cold, or samples allowed to remain in acetone for longer than 2 h. Studies on storage of resolubilized proteins were also performed in the presence and absence of protease inhibitors. Sample aliquots were compared by focussing immediately, by being kept at room temperature for 1 week after resolubilization, and by 1 week of liquid nitrogen storage after resolubilization, before focussing. Any storage resulted in smeared spots, showing clearly that the resolubilized proteins must be focussed as a fresh solution (unpublished data).

IPG strips require loading of samples onto the strip. The samples can be either loaded at one location (cup loading) or the sample can be included in the rehydration step. We found that cup loading for fruit proteins at the cathode gave the best results compared to in-sample rehydration which gave unfocussed smears, under the same focussing conditions. In addition to the method of loading, long focussing times are extremely important for plant protein preparations. Early work used the focussing guidelines in the Amersham 2D booklet. These gave bands rather than spots, indicating a lack of focussing. A series of experiments was conducted where the focussing times were increased until optimal focussing occurred. These final guides are given in Table 1. We also believe that the electrode wicks used with the MultiPhor system are important to the success of our method

for plant 2-DE work. The wick acts to remove salts and other interfering compounds from the proteins so that they do not interfere with the protein focussing. The techniques described here appeared reproducible and robust, and have worked well in both our laboratories.

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